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**Table of Contents**

## Abstract

The EGF receptor family consists of four members (EGFR and HER 2, 3 and 4). One, HER2, is overexpressed in 20-25% of human breast cancers through gene amplification while another, the EGF receptor, is thought to be overexpressed or activated in poor prognosis breast cancers. Our objective was to determine whether the most recently discovered member, HER4, triggers a distinct, anti-proliferative and differentiation signal in breast cell lines and could therefore be a marker breast cancer with a better prognosis. After cloning the HER4 cDNA and constructing various chimeric and mutant receptors, we created novel breast cell lines expressing a variety of molecular constructs. These were used to conclusively demonstrate that HER4 activation stimulated anti-proliferative and differentiation responses in breast cell lines, even in the absence of HER2 signaling. Thus HER4 is both necessary and sufficient to slow the growth of breast cancer cell lines. Studies in our extension year will define gene expression by microarray technology, delineating HER4 versus combined HER2 HER4 signals.

## Introduction

The human EGF receptor 2 gene (HER2) is amplified and overexpressed in 20-25% of invasive breast cancer (1). Moreover, many (but not all) observations indicate that poor prognosis breast cancers exhibit increases in EGF receptor content and/or an EGF receptor autocrine loop with the production of TGFa (2,3 and see Table 1). Both EGF receptor and HER2 can interact with each other, or with HER3 leading to growth and proliferation (4,5). Ligand (heregulin) dependent or independent activation of HER3 and HER2 signaling may be responsible for a proliferative signal. However, in certain cells, heregulin causes differentiation. It is possible that this heregulin-dependant differentiation is a product of HER2 signaling, but it may also involve signaling through the fourth member of the family HER4 (6,7). A third possibility is that in selected cells HER4 and HER2 hetero-dimerize and a combined HER2/HER4 signal causes heregulin-dependant slowing of growth and induction of differentiation. If HER4 signals stop proliferation and cause differentiation, then HER4 activation may slow the growth of breast cancer.

In addition, any HER4 anti-proliferative pathway would contain gene products with anti-growth functions that could be classified as tumor suppressors. Loss of such tumor suppressors could be a cause of breast cancer progression. Our tasks in this DOD grant were to obtain definitive evidence that HER4 provides a different biologic signal in breast epithelium, i.e., differentiation and/or anti-proliferation rather than proliferation, and to elucidate the pathway, or elements of the pathway, that differ between HER4 and the original three members of this receptor family (EGF receptor, HER2 and HER3). To this end, we created molecular reagents, and cell lines and devised technology which allowed us to prove that HER4 sends an anti-proliferative signal. These reagents and cell lines should provide us the wherewithal to isolate the unique members of the HER4 pathway as well as substrates phosphorylated by this tyrosine kinase in the coming years.

## Body

Using this DOD funding, we have firmly established that HER4, as a unique signaling agent, is

capable of sending an anti-proliferative signal in breast epithelia cell including at least some breast cancer cell lines. In addition, HER4 is at least partly responsible for a differentiation signal including the synthesis of neutral lipids and the induction of E-cadherin, a transmembrane protein whose expression decreases in many aggressive breast cancers. To reach these conclusions, we have created multiple molecular constructs, several antibodies, and a number of unique, stably transfected cell lines. These will allow us to pursue subsequent objectives in the future, the elucidation of the signaling pathway by which HER4 sends its anti-proliferative and differentiation signals.

**A. The creation of molecular reagents to study the growth promoting and differentiation effects of EGF receptor family members.**

Full-length HER4 cDNA clones, to our knowledge, were only available by materials transfer agreements with one of several companies. These type of agreements inhibit the free flow of information and we thought it important to make our own reagents. To this end, we cloned the cytoplasmic domain of the HER4 receptor and created a EGF receptor (extracellular and transmembrane domains) HER4 (cytoplasmic tyrosine kinase domain) chimera. We then decided for a number of reasons that we needed the entire HER4 molecule and therefore we isolated by PCR the HER4 extracellular domain again using RNA isolated from the MDA-MB 453 cells. After high fidelity PCR and exhaustive sequencing, we selected a clone that was wild type with respect to amino acid sequence and created a full length HER4 cDNA expressed using two vectors pcDNA and pLXSN. The latter can be used to make amphotrophic viruses capable of infecting human breast cell lines, and in fact we used that strategy several times (see below). **Figure 1** schematically outlines clones and shows our strategy, which we have now accomplished, to create a kinase-dead full length HER4 and a HER4 kinase dead EGF receptor/HER4 chimera. These constructs were needed to achieve the aim of defining a HER4 signal. We showed that the chimera was capable of slowing growth both in the breast cancer cell lines and in 32D cells (see below). The appropriate control to show that this was due to the HER4 tyrosine kinase is a kinase-dead construct.

Another type of dominant negative construct was made, the extracellular domain anchored by a transmembrane domain of HER4. We've used the full length HER4 cDNA and placed a stop codon right after the tri-basic amino acid anchoring sequence in the juxtamembrane area of the molecule. High level expression could block the differentiation effect of heregulin in MDA-MB 453 cells and other cells in which heregulin causes differentiation. These experiments are planned.

Lastly in the area of creation of new reagents, we realize that since the HER4 signal is growth inhibitory it may be difficult to create cell lines expressing this molecule. Therefore we cloned the full length HER4, the chimera, as well as the kinase dead version of these two molecules and the HER4 dominant negative cytoplasmic domain into the tetracycline off system (**Fig 2**). This was felt to be one the best inducible promoter systems. However, we have not been successful in creating tet responsive gene expression in any of the breast cancer cell lines that we tried. The background expression remained high, the system was therefore not inducible. Fortunately, we were able to make new cell lines stably expressing HER4 (see below).

## B. The Action of the EGF Receptor HER4 Chimera and 32D Cells

We created stably transfected neo-resistant 32D cell expressing the EGFR:HER4 chimera. Populations of chimeric transfected 32D cells underwent growth slowing in response to ligand (EGF). This was in line with our hypothesis that HER4 sends a differentiation and not a proliferative signal. We next selected two clones, which express different levels of the HER4 chimera. EHC-2 expresses a high level and EHC 11 expresses 10-20% as much HER4 chimera. **Figure 3** shows a FACS analysis of HER4 chimera expression. **Figure 4** shows the amount of tyrosine phosphorylated HER4 chimera in the two clones. The high expressor, EHC 2, exhibits constitutive autophosphorylation of HER4. The addition of EGF dramatically increases tyrosine phosphorylation but the most important aspect is that there is phosphorylation in the basal, non-ligand state. This correlates with the growth curve **Figure 5**, which demonstrates that EHC2 grows much more slower and that EGF blocks IL-3 dependent growth in this clone. The lower expressor, EHC clone 11, contains HER4 chimera and it can be activated in a EGF-dependent manner. However, there is no constitutive phosphorylation and this clone grows much faster (as shown in **Figure 5**).

## C. Production of Antisera

We have not been satisfied with the commercial antisera to HER4 (or HER3) and therefore made polyclonal antibodies by creating GST-fusion proteins and contracting with a commercial vender to produce antibodies. An original GST HER4 fusion protein immunization using the mid C-terminal region did not produce antisera with significant titer. We next re-immunize with a 100 amino acid C-terminal fragment of HER4 and have now obtained an excellent immunoprecipitating antibody. **Figure 6** shows that HER4 antibody will immunoprecipitate tyrosine phosphorylated HER4 from clone 2. The antisera is sufficient to detect phosphorylated HER4 in ligand treated breast cell lines that express modest HER4 levels. We have recently obtained anti-HER3 antibodies with excellent immunoblotting properties using a GST HER3 C-terminal fusion protein as immunogen.

In addition, we have placed the extracellular domain of HER4 into the p-FASBAC vector and have created a HER4 extracellular domain baculovirus (**Fig 7**). This soluble protein has the 6 histidine tag at the end and therefore we can purify the extracellular domain using nickel columns. This immunogen was used by a contractor in an attempt at monoclonal antibody production. Our aim was to create a monoclonal antibody capable of recognizing the human HER4 in paraffin-embedded sections. In this manner, we would be able to do translational research with archived tissues allowing us to determine whether the HER4 expression level is an independent prognostic variable for breast cancer survival or whether it is a modifier of HER2 predicted prognosis. Unfortunately, our contractor was not successful in making monoclonal antibodies. We have obtained as a gift of Mark Slikowski of Genentech, several candidate HER4 monoclonals that we will test for the desired properties.

## D. Studies of the SUM 44 Cell Line

We work with a number of breast cancer cell lines, including those initiated in Steve Ethier's

laboratory at the University of Michigan. **Figure 8** shows that treatment of various cell lines with heregulin resulted in growth suppression in several lines including the SUM 44 cell line. The MDA-453 cell line which has been shown by others to exhibit heregulin-dependent differentiation does not do so in our hands. Investigation showed that our clone has a high level of basal (non-ligand-dependent) HER4 tyrosine phosphorylation. The cell line grows slowly due to a heregulin autocrine loop. Our work analyzing HER4mRNA (see later section) shows that only cells that express HER4 mRNA undergo heregulin-dependent growth slowing. **Figure 9** shows the SUM44 anti-proliferative response to both heregulin and HB-EGF. Both ligands (from two separate ligand families) are capable of activating HER4 tyrosine phosphorylation. **Figure 10** shows the extent of HER4 tyrosine phosphorylation in SUM 44 and MDA 453 cells treated with heregulin or HB-EGF. It is clear that heregulin stimulates a HER4 signal as a judge by tyrosine phosphorylation to a greater extent than HB-EGF. These experiments were accomplished using our polyclonal HER4 antisera. This antiserum is excellent for immunoprecipitation and western blotting and, we believe, is superior to any commercial antibody on the market. **Figure 11** shows that SUM44 cells treated with heregulin differentiate as judged by morphology and their elaboration of neutral lipids indicating that the HER4 tyrosine phosphorylation results in a true differentiation phenotype. **Figure 12** shows a fluorescence activated cell sorter analysis of SUM44 cell neutral lipid content which increases with heregulin treatment. Other assays (not shown) demonstrate that DNA content increases in cells treated with heregulin. Cells enlarge as they differentiate, as well as double their DNA content. This could be interpreted as cell cycle arrest at the G2M border, but it could also be a result of polypliod development (endoreduplication) in differentiated breast cell line. Lastly, stable transfection of the kinase dead HER4 cDNA into SUM44 cells blocked heregulin-dependent growth slowing (**Fig 13**). This shows that HER4 is necessary for the ligand-dependent anti-proliferative response.

**E. Development of a Matched Pair of Cells Expressing or not Expressing HER4.**

To give additional proof that the HER4 signal is anti-proliferative, we created matched pair of transfected cells, which expressed or did not express HER4. We screened available cell lines and determined that the SUM102 cell line did not express the HER4 receptor. Our HER4 molecular constructs (and vector alone conferring neo resistance) were packaged as an amphotrophic retrovirus and used to infect several hard to transfect cell lines such as the SUM 102 cell. G418-selection resulted in the creation of two types of cells, SUM 102-HER4 and SUM 102 neo vector. **Figure 14** provides convincing evidence in SUM 102 neo vector cells did not exhibit HER4 activation when treated with heregulin. On the other hand, clones were selected in which heregulin dramatically stimulated HER4 tyrosine autophosphorylation. Thus, we have created a matched pair of cell lines expressing HER4 or vector alone.

These cells were tested for their growth inhibititory response. **Figure 15** demonstrates an anti-proliferative response of HER4 containing cells to heregulin. The cells (SUM102 HER4) also show an anti-proliferative response to HB-EGF (not shown). **Figure 16** shows the results of the neutral lipid FACS assay we have developed. The cells treated with or without heregulin were stained with Nile Red and then analyzed by FACS. Heregulin produced a significant shift in Nile Red positive cells providing evidence that not only did heregulin slow the growth of the cells expressing HER4, but heregulin resulted in differentiation. As expected, control SUM 102

neo vector cells did not respond to heregulin with differentiation. Another assay for differentiation is the induction of E-cadherin. This gene is not expressed in SUM44, so we could not assess the ability of a HER4 ligand to induce E-cadherin in those cells. However, in transfected SUM102 HER4 cells, heregulin increased E-cadherin expression; this did not occur in neo vector SUM102 cells (**Fig 17**).

**F. Demonstration that HER4 sends an anti-proliferative signal in the absence of HER2.**

It has been known for over 10 years that when certain cells are treated with heregulin they differentiate, in fact, one of the original groups that isolated a putative ligand for HER2 called the protein, the neu differentiation factor or NDF because the cell systems that they studied differentiated when treated with the factor during purification (8). Virtually all of the studies investigating heregulin-dependent cell growth or differentiation using breast or ovarian cell lines suffer from the same problems. Cells that differentiate express both HER4 and HER2, making it difficult, if not impossible, to distinguish whether HER4 by itself slows growth or causes differentiation.

To address this, we have adopted a technology pioneered by Nancy Hynes (9). She has taken a single chain antibody to HER2 fused to an endoplasmic reticulum (ER) targeting sequence. The expression of this intracellular, single chain antibody results in trapping HER2 intra-cellularly before it can be transported to the plasma membrane where it could signal in a heregulin-dependent manner. We obtained the sc anti HER2 antibody from Dr. Hynes in a vector, which encodes resistance to the antibiotic puromycin. We created two matched pairs of cell lines. The first was the SUM 44 cell, which expresses both HER4 and HER2. Transfection of cDNAs encoding either vector or vector plus containing scHER2 antibody into SUM 44 followed by selection created two populations of puromycin resistant cell lines. **Figure 18** shows that the addition of heregulin to the vector alone expressing cells results as expected in heregulin-dependent HER2 phosphorylation, as shown by HER2 immunoprecipatation followed by p-tyr immunoblotting. However, the addition of heregulin to the cell line expressing the single chain antibody can not stimulate HER2 tyrosine phosphorylation because HER2 was not at the cell surface (**Fig 18**). We have done immunofluorescence and have shown that the reason why HER2 was not activated was because all the HER2 was found intracellularly, not on the cell's membrane (data not shown). Thus, we have successfully abolished HER2 signaling in these cells.

None the less, addition of heregulin to cells without a HER2 signal produces an anti-proliferative effect as does the addition of HB EGF in SUM44 (**Fig 19A**). As in all other situations, HB EGF is less effective than heregulin in creating anti-proliferative response, because it does not stimulate HER4 as well as heregulin. This shows that heregulin-dependent anti-proliferations does not involve HER2 and that the HER4 signal alone is sufficient. The latter follows because HB-EGF, which would not trigger HER3 tyrosine phosphorylation, also slows cell growth.

An even more complex set of cell lines were created by infecting the neo-resistant SUM102 HER4 cells (containing the neo-resistant vector or the vector encoding both neo and HER4) with a second cDNA encoding either puromycin-resistance alone, or puromycin-resistance plus the single chain HER2 antibody. This was accomplished by packaging retroviruses with the

puromycin resistance vectors and infecting cells. The cells were then selected using both selectable markers (G418 and puromycin). Once the cell lines were created, we were able to show that heregulin-dependent, anti-proliferative response was retained when HER2 signaling was abolished (**Fig 19B** SUM102-HER4-5R). The results are conclusive; a heregulin-dependent, anti-proliferative effect is fully operable in the absence of any HER2 signaling.

The production of neutral lipids in SUM44 and SUM102 cells and the induction of E-cadherin in SUM102 cells, all markers of differentiation, are less well induced in the cell lines without cell surface HER2 signaling (the SUM44 and SUM102 5R bearing cells). However, this is in part because the basal level of lipids goes up with the withdrawal of cell surface HER2 i.e HER2 may contribute to the undifferential state. Thus we can say reasonably that HER4 is necessary and sufficient for an anti-proliferative signal, and that HER4 is at least necessary for the differentiation signal. However, HER4 might not be totally sufficient for all aspects of differentiation.

Results showing the HER4 is necessary are confirmed in SUM44 cells in which we have transfected dominant negative kinase dead HER4 constructs (see **Fig 13**). This abrogates the HER4 signal and prevents the anti-proliferative response. This is an area in which we will continue to work. We will try to tease apart a potential role of hetero-dimerization between HER4 and HER2 signals in the differentiation response as opposed to the HER4 alone sending an anti-proliferative signal. This next year we will use our extension to perform microarray analysis of gene expression using cells with a "pure" HER4 signal versus a mixed HER4 HER2 signal.

As a part of these studies, we have demonstrated that heregulin-dependent, HER4 activation in the absence of HER2 is perfectly capable of sending activating MAP Kinase and AKT. These experiments are the beginning of our analysis of pathways directly stimulated by HER4.

### G. Quantitative PCR for HER4 mRNA Levels

A long term objective of this project is to understand the potential of HER4 as a marker of breast cancer prognosis and perhaps even response to therapy. Multiple translational studies have attempted to measure the amount of HER1 (EGFR) and particularly HER2 expression in breast cancer. It is widely accepted that the HER2 gene amplification occuring in ~20% of women with breast cancer is both a poor prognostic sign and a finding that has therapeutic import. The roles of HER1, HER3, and HER4 as individual entities are much less well defined and activated receptor signaling as a predictive function is even harder to approach.

There are multiple articles describing the expression of EGF receptor and its relationship to prognosis in breast cancer (see Table 1). These data in the aggregate are confusing and certainly not definitive. Studies of HER3 and HER4 expression are fewer and in general, have not been done on large data sets (10-12). Several of the small clinical studies of HER4 expression favor the idea that HER4 is a good prognostic sign;(12) this would support the central hypothesis of this proposal that HER4 is a differentiation signal. This finding is compatible with data from our group and others. Since EGF receptor family members heterodimerize and the outcome of the signals may depend upon the partners heterodimeric complex (eg. HER2/HER3 vs.

HER2/HER4), it is important to analyze the expression of all 4 family members in breast cancers to fully understand the family's biology and prognostic import. This is a daunting task. The best method would be to have antibodies to all four members that are useful in archival, paraffin-embedded samples. Good antibodies for all members are not available. Thus, while there are issues regarding the interpretation of the relationship between mRNA levels and protein levels, we have chosen to use new quantitative PCR methods for accurately quantitating family member mRNA levels (ABI 7700, "Taq man").

Our experience shows that the quantitative PCR assay is extraordinarily accurate and reproducible once one has extracted and accurately measured RNA. During the last year, we have created PCR primers and specific "Taq man" probes for the EGF receptor, HER2, HER3, and HER4. We have cloned the 4 human family member cDNAs into vectors that allow us to transcribe RNA from the vector to make more mRNA populations that can be used to develop standard curves for the Taq man assay (**Fig 20**). We have developed accurate, highly reproducible, standard curves for each of the four molecular species. We have miniaturized the assays so that we can use very small (ng) amounts of total RNA (without the need for poly A selection) to measure mRNA content of HER1-4. **Figure 20** shows representative standard curves. **Figure 21** demonstrates the HER4 mRNA expression in a panel of breast cell lines that we have examined. One of those lines, SUM44, was used for multiple experiments.

SUM44 expressed reasonable HER4 levels without exhibiting non-ligand-dependent autophosphorylation. **Figure 22** shows the levels of HER1-4 in a panel of cell lines. It is interesting to note that all cells express HER3. HER3 levels are always greater than HER4 levels in this panel of cell lines.

With other funding, we are beginning to use quantitative PCR to assess EGFR family member mRNA levels in human breast cancers. We have extracted mRNA from over ~30 matched pairs of operative samples in which breast cancers and matched "normal" tissue adjacent to the tumor was obtained. We are also working on extracting RNA from breast cancer core biopsies, so that we can follow EGF receptor family mRNA expression before and after chemotherapy in our neo-adjuvant protocols. This particular DOD grant will end before these human studies are begun, but the current funding has funded the development of a technique for HER4 mRNA assessment.

## H. Key Research Accomplishments.

- Cloning of full length HER4 cDNA
- Cloning of a chimeric EGF receptor (extracellular and transmembrane domains) HER4 (cyloplasmic domain cDNA)
- Site directed mutagenesis of above cDNAs to abolish tyrosine kinase activity creating kinase dead, dominant negative constructs
- Creation of retroviral vectors expressing HER4 and dominant negative HER4 constructs
- Creation of polyclonal antisera against the C terminis of human HER4 and HER3
- Creation of a his-tagged baculovirus expressing HER4 extracellular domain
- Creation of 32D myeloid cell lines expressing the EGF receptor HER4 chimera

- Demonstration that the EGFR HER4 chimera 32D line slows growth in response to ligand
- Demonstration of heregulin-dependent HER4 tyrosine phosphorylation in some breast cancer cell lines
- Demonstration that heregulin-dependent breast cell line differentiation only occurs in HER4 expressing cells
- Creation of matched pair of cell lines expressing vector or full length HER4 from a cell line, SUM102, that did not express HER4
- Demonstration that HER4 signaling in a cell line made to express HER4 causes differentiation
- Creation of a breast cell lines expressing an EGFR:HER4 chimera and demonstrating EGF-dependent differentiation
- Creation of cell lines with dominant negative (kinase dead) HER4 and demonstrating that this dominant negative construct blocks heregulin-dependent differentiation in a HER4 expressing line
- Creation of a new flow cytometry assay to assess neutral lipids in differentiating breast cell lines
- Demonstrating that heregulin-dependent HER4 activation upregulates E-cadherin, an additional assay of HER4-dependent differentiation
- Creation of pairs of cell lines expressing either vector (p BABE) or a cDNA expressing a singe chain (sc)HER2 antibody with an endoplasmic reticulum (ER) targeting sequence
- Demonstrating that expression of scHER2 antibody with an ER targeting sequence prevents cell surface expression of HER2 and heregulin-dependent HER2 activation
- Demonstrating that in the absence of HER2 signaling heregulin still has an antiproliferative effect in cells with HER4 signaling. This demonstration meets one of the two major objectives of this grant
- Demonstration of heregulin-dependent MAPK and AKT activation in HER4 expressing cells even in the absence of HER2 signaling
- Creation of an ultrasensitive quantitative PCR assay for HER4 mRNA levels and a similar assay for EGFR, HER2 and HER3 RNA levels
- Use of the quantitative PCR to measure the level of HER1-4 mRNA in a panel of breast cancer cell lines
- Demonstrating that only cells expressing HER4 and not expressing autocrine heregulin pathways exhibit a heregulin-dependent antiproliferative effect
- Initiation of studies of quantitative PCR in samples derived by laser capture microdissection.

**Section 8-Outcomes**

1. Sartor, Carolyn I., Zhou, H., Koslowska, E., Guttridge, K.I., Kawata, E., and Earp, H.S. Role of HER4 in Differentiation of Human Breast Cancer Cells. Proceedings of the American Association for Cancer Research 41:67, 2000.

2. Earp, H.S., Zhou, H., Koslowska, E., Guttridge, K., Kawata, E., Ethier, S.E., Sartor, C. HER4 Signaling and Breast Cancer Cell Differentiation. Era of Hope Department of Defense Breast Cancer Research Program Meeting Proceedings 1:402, 2000.

3. Sartor, C.I., Zhou, H., Koslowska, E., Guttridge, K., Kawata, E., Calvo, B., Caskey, L., Ethier, S., Earp, H.S. HER4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells. In preparation.

**Section 9-Conclusions**

We have demonstrated that HER4 signaling is both necessary and sufficient to deliver a ligand-dependent, anti-proliferative signal. These conclusions have been reached with members of two different ligand families and at least two human breast cancer cell lines. The majority of breast cancer lines do not express HER4 and our hypothesis is that its anti-proliferative effect is selected against when breast cancer cells are forced to grow out as established lines in culture. In fact, HER4 may be lost in breast cancer progression for the same reason. We have made multiple molecular construct for those studies and used these to create new cell lines that can be used to study a "pure" HER4 signal in the absence of HER2, or a combine HER4,HER2 signal. We will use the models created during the next year's extension, to perform more indepth studies of HER4 signaling in the presence and absence of HER2. We intend to perform microarray "geneochip" analysis on these cell lines to detect the families of genes expressed in response to the anti-proliferative HER4 signal. This will allow us to approach a secondary objective which, is to determine whether there are any tumor suppressor genes in HER4 anti-proliferative signaling pathway that can be used along with HER4 expression as prognostic variables in predicting the outcome of breast cancer and its therapy.

In addition, we have set up a quantitative PCR assay for HER4 so that we can accurately assess the level of mRNA expression for HER4 (as well as the EGF receptor, HER2 and HER3). Multiple laboratories have shown combinatorial interaction between these receptors and their ligand families. A full understanding of breast cancer prognosis and biology will only be obtained when we know the expression and, in fact, the level of activation of the multiple family members. Our quantitative PCR assay is being miniaturized, and we hope to be able to use laser capture microdissected material during the next year to assess accurately EGFR and HER2, 3 and 4 expression in breast cancer samples versus adjacent normal breast epithelium.

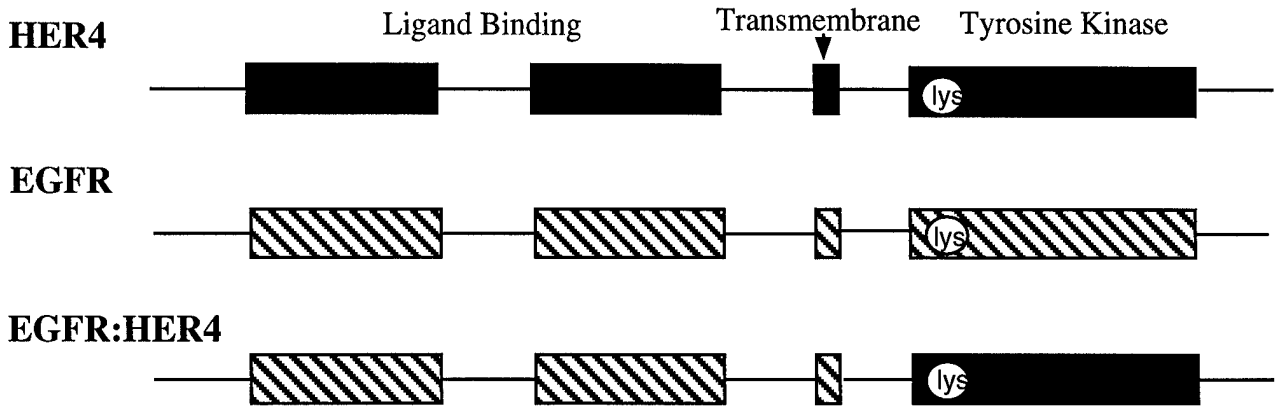
In summary, we have met one major objective by determining that HER4 is indeed an anti-proliferative and differentiation signaling receptor. We have created the molecular and cellular reagents to allow us to dissect a pathway of anti-proliferation and potentially detect tumor suppressor genes. Lastly, we have created a quantitative PCR assay using breast cell lines. In the future, this assay will allow us to screen breast cancers in clinical translational studies to determine whether our overall hypothesis regarding the biology of the HER4 receptor is reflected in the prognosis of breast cancers expressing HER4.

## Table 1

## Epidermal Growth Factor Receptor (HER1) Levels in Breast Carcinomas and Related Outcomes Analysis

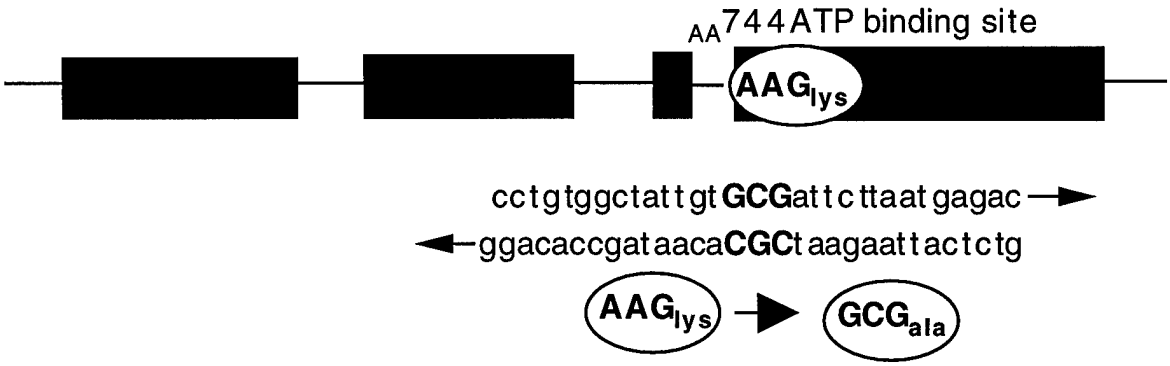
| Author | Year | N | Method | Cut-Off | % Pos. | Univ. RFS | Univ. OS | Multi Var. RFS | Multi.Var OS |
|---|---|---|---|---|---|---|---|---|---|
| Rios[13] | 1988 | 179 | LB | 1 fmol/mg | 43 | <0.05 | | | |
| Costa[14] | 1988 | 376 | LB | 10 fmol/mg | --- | | | | |
| Grimaux[15] | 1989 | 55 | LB | 5 fmol/mg | 33 | NS | 0.05 | 0.01 | <0.01 |
| Foekens[16] | 1989 | 203 | LB | None | 91(any +) | NS | | NS | |
| Spyratos[17] | 1990 | 109 | LB | 10 fmol/mg | 34 | 0.05 | | 0.03 | |
| Lewis[18] | 1990 | 90 | IH | >2+ | 14 | <0.003 | | 0.04 | |
| Toi[19] | 1991 | 135 | LB | 1 fmol/mg | 41 | <0.05 | | | |
| Hawkins[20] | 1991 | 120 | LB | 1 fmol/mg | 43 | NS | NS | NS | NS |
| Nicholson | 1991 | 231 | LB | 10 fmol/mg | 35 | <0.001 | <0.001 | 0.03 | NS |
| Osaki[22] | 1992 | 115 | LB | 1 fmol/mg | 35 | <0.01 | | | |
| Bolla[22] | 1992 | 232 | LB | 3 fmol/mg | 51 | NS | | NS | |
| Shrestha[23] | 1992 | 50 | IH | Any | 44 | <0.05 | <0.05 | | |
| Gasparini[24] | 1992 | 164 | IH | >5% cells | 56 | 0.003 | NS | 0.0049 | |
| Fox[25] | 1993 | 370 | LB | 20 fmol/mg | 47 | 0.03 | 0.05 | 0.03 | NS |
| Koenders[26] | 1993 | 376 | LB | 50 fmol/mg | 22 | 0.03 | 0.002 | NS | NS |
| Murray[27] | 1993 | 107 | mRNA | + or ++ | 51 | NS | NS | | |
| Hawkins[28] | 1996 | 215 | | | | NS | NS | NS | NS |

**LB** = ligand binding. **IH** = immunohistochemistry. **Univ**= univariate. **RFS** = Recurrence Free Survival. **OS** = Overal Survival.




# HER4 and EGFR:HER4 kinase dead

## HER4 kinase dead




## EGFR:HER4 kinase dead


**Figure 1. Expression vectors for use in HER4 experiments.** We isolated the HER4 extracellular domain and constructed a full length HER4 cDNA. In addition, we created two kinase dead mutants by site-directed mutagenesis.

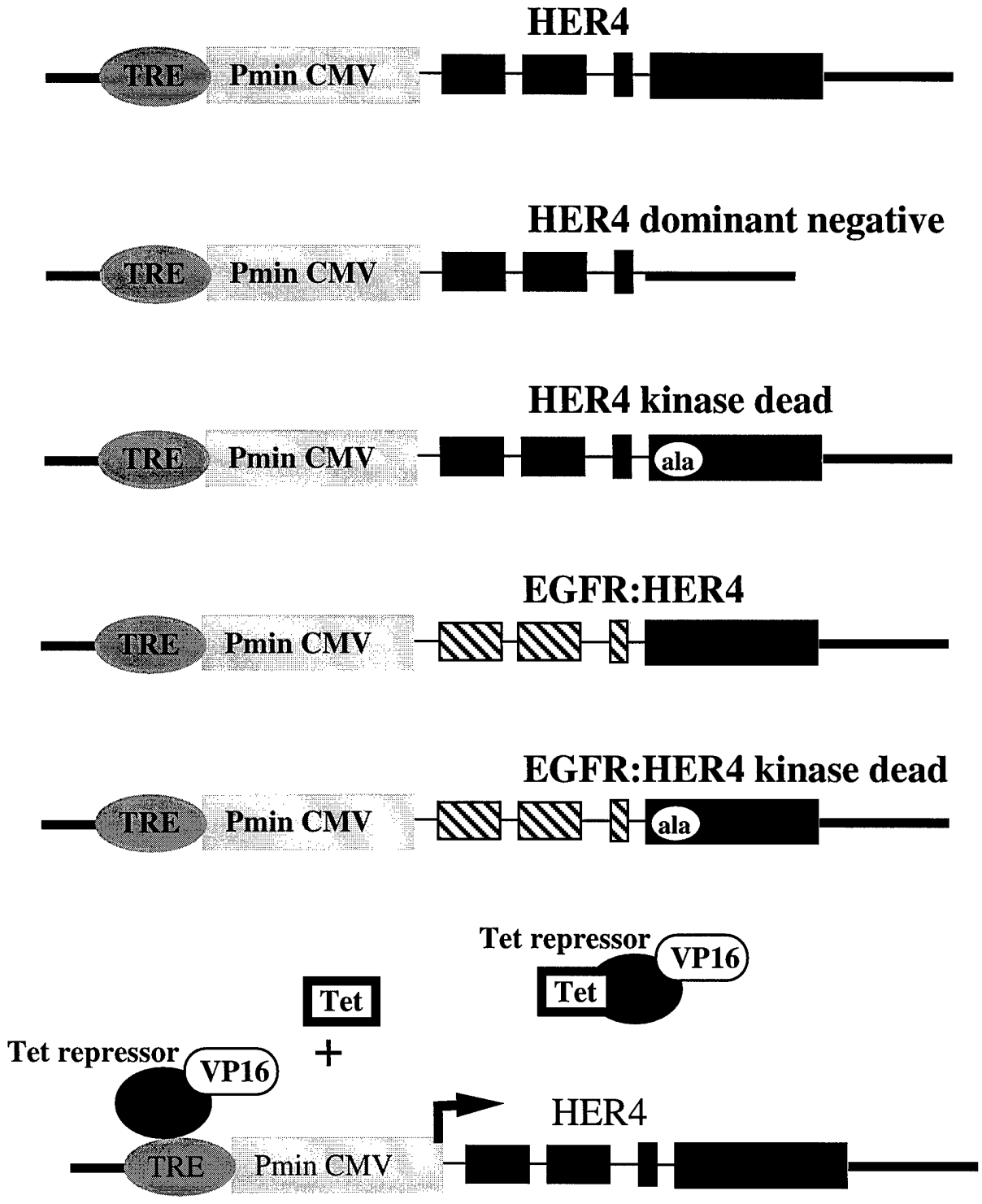



**Figure 2. Inducible promoter for HER4 constructs.** Each of the important HER4 cDNAs were placed in the "Tet off" vector which would allow the creation of stable transfected lines with tightly-controlled HER4 construct expression.

**vector control**

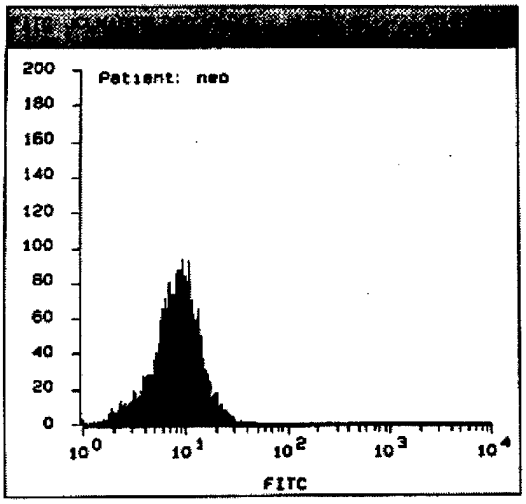
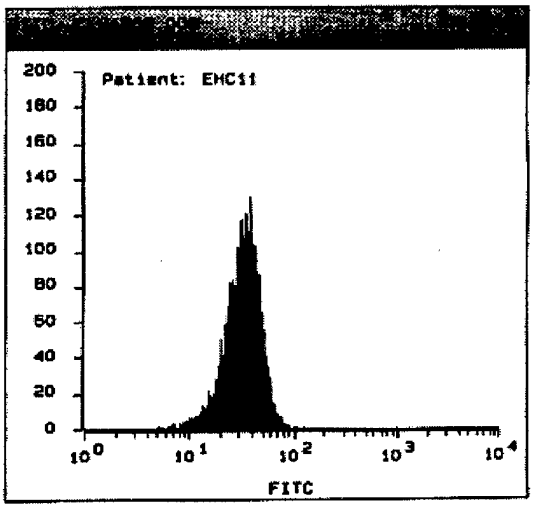
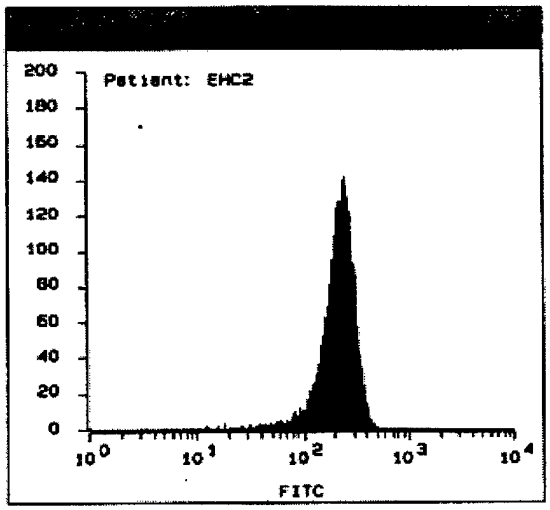



**Figure 3. Fac Scan analysis shows that we have created two 32D cell clones with constitutively high (EHC2) and low (EHC11) levels of expression.** 32D cells were washed and incubated with anti-EGFR antibody, followed by an incubation with anti-rabbit FITC conjugated secondary antibody. The cells were washed once more and analysed by flow cytometry for FITC staining.

**EGFR:HER4 clone 11**




**EGFR:HER4 clone 2**



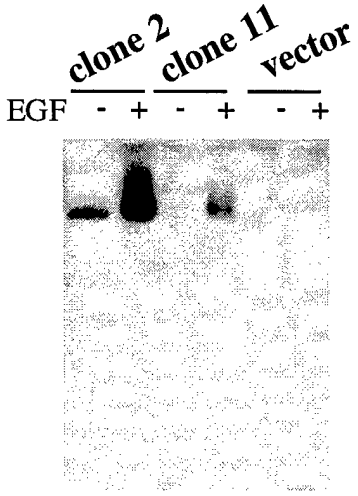



**Figure 4. The high (EHC2) and low (EHC11) expressing clones exhibit different levels of ligand-independent (-EGF) and ligand-dependent(+EGF) tyrosine phosphorylation.** 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 μg/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-EGFR antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. Clone 2 expresses the EGFR:HER4 chimera at 5-10 times the level of clone 11. The chimera is constitutively activated but can be further stimulated by EGF. Whereas clone 11 expresses the chimera to a lower level and is active only with the addition of EGF.

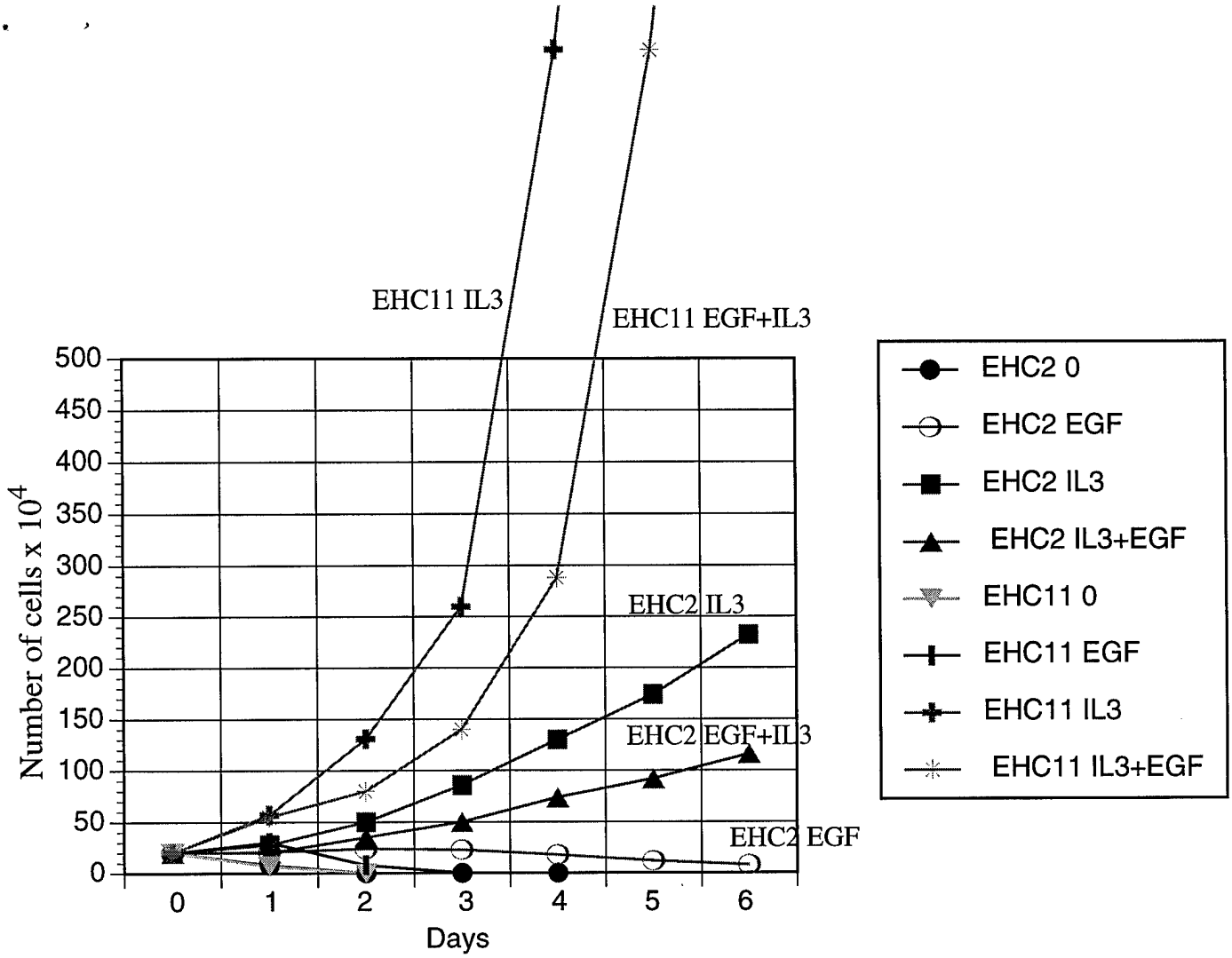



**Figure 5. Growth curves of EHC expressing 32D clones.** High expressing EHC2 clone shows a different growth pattern compared to low expressing EHC11 clone. EHC11 cells grow exponentially with the addition of IL3, and slightly slower with both IL3 and EGF. With no addition or addition of EGF alone the EHC11 cells die quickly; all are dead within two days. Thus the level of HER4 chimera expression in EHC11 is insufficient for biological signalling. In contrast, EHC2 which expresses EHC at high levels and exhibits an autoactivated HER4 kinase, grows slowly in the presence of IL3, and even slower with the addition of both IL3 and EGF. However, addition of EGF prevents cell death, and EHC2 cells remain viable many days after EHC11 cells have died.

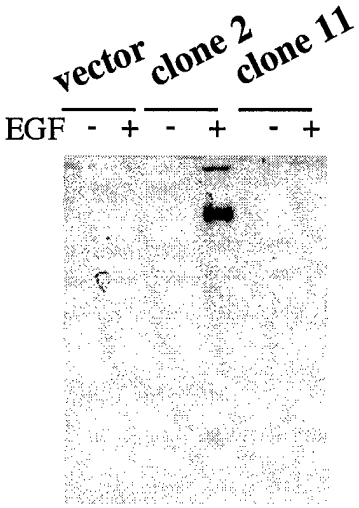



**Figure 6. A GST-HER4 fusion protein was used to raise anti-HER4 antibody.** 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 μg/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-HER4 antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. In addition, when the blot is overexposed a faint band is seen with clone 11. Thus we have a specific anti-HER4 antibody.

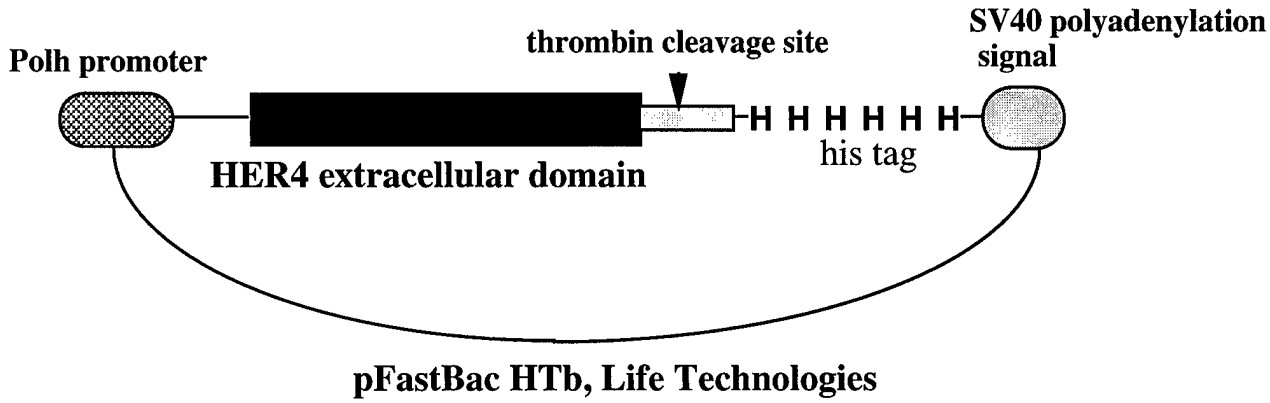



**Figure 7. Creation of an extracellular domain HER4 baculovirus.** HER4 extracellular domain was cloned into pFastBac baculovirus expression plasmid. Purification with $Ni^{++}$ columns that bind to the 6-His tag will provide immunogen for monoclonal antibody formation.

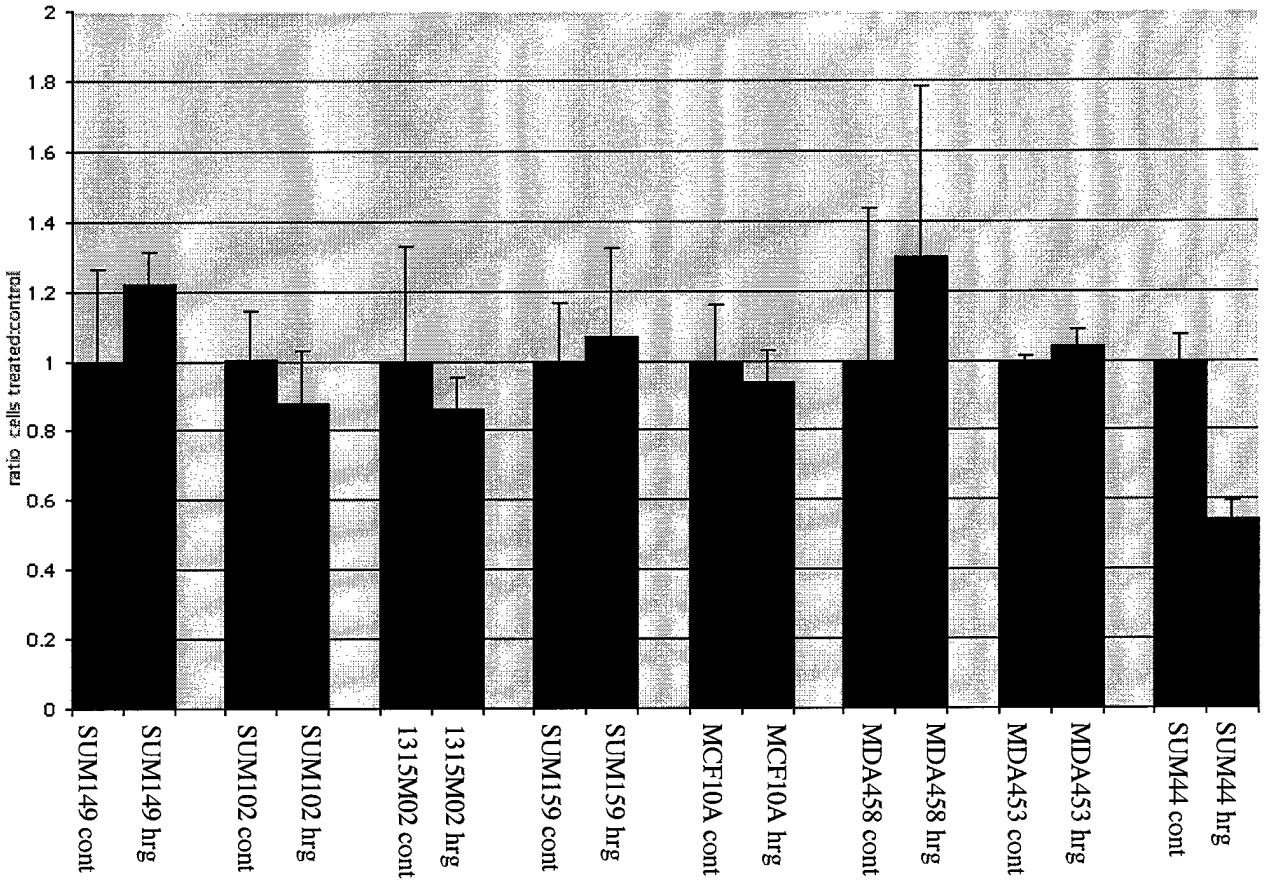



**Figure 8. Proliferative response of human breast cancer cell lines to heregulin.** Cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of heregulin is shown. Error bars represent standard deviation of at least 3 experiments. SUM44 cells demonstrated the most pronounced effect of heregulin, an antiproliferative effect.

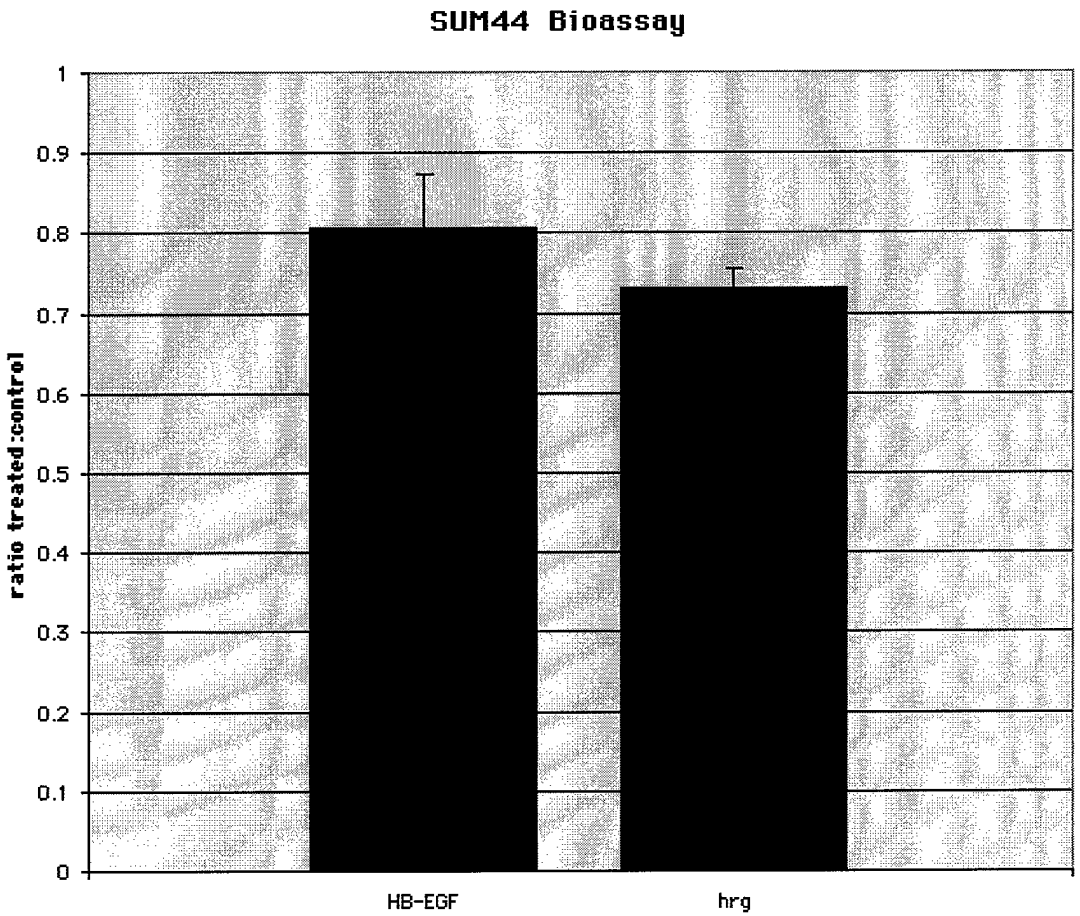



**Figure 9. Anti-proliferative response to HB-EGF.** SUM44 cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 or 100 ng/ml HB-EGF for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. Like heregulin, HB-EGF caused an anti-proliferative effect, although not to as great a degree.

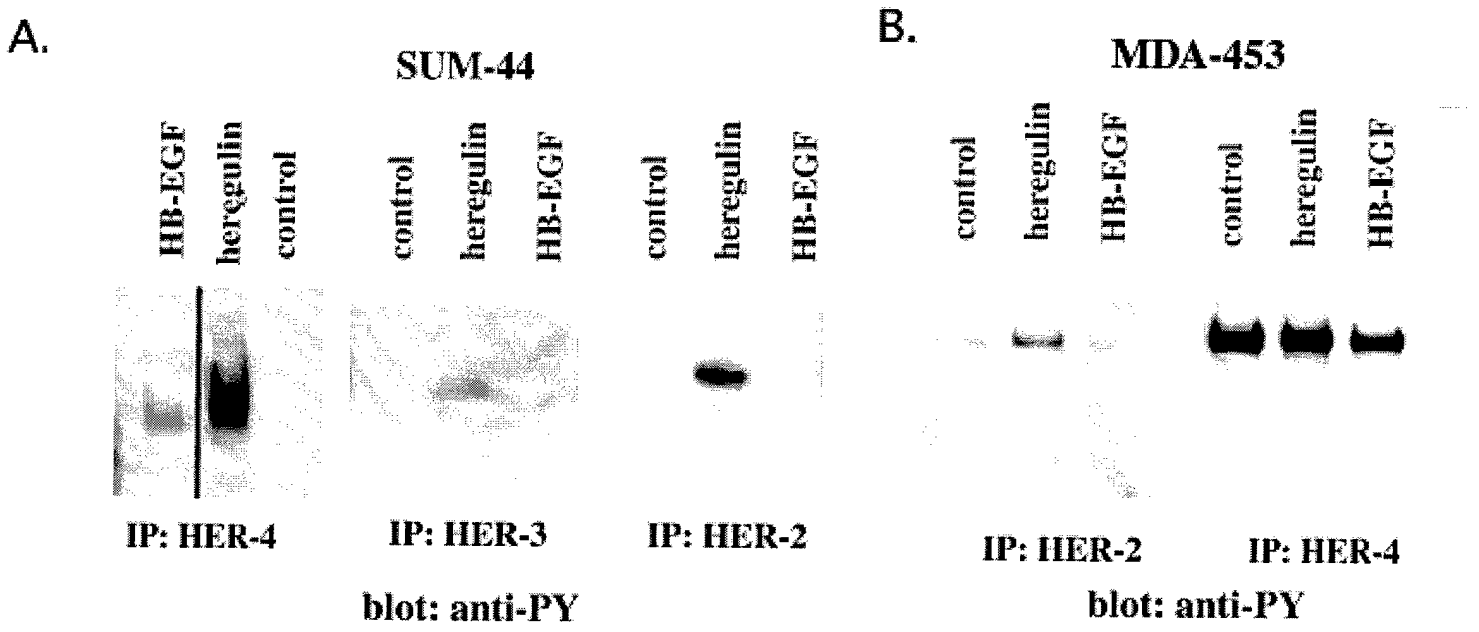



**Figure 10. HER2-4 tyrosine phosphorylation in response to heregulin stimulation.** SUM44 cells (A) or MDA-453 cells (B) were treated or not with 10 ng/ml heregulin B1 or 100 ng/ml HBV-EGF for 30 m inutes. Cell lysates were immunopreciptated with anti-HER2, HER3, or HER4 and immunoblotted with anti-phosphotyrosine. Heregulin induced tyrosine phosphorylation of HER2, HER3, and HER4 in SUM44 cells. Heregulin induced tyrosine phosphorylation of HER2 and HER3 in MDA453 cells, but these cells demonstrated constitutively phosphorylated HER4, which was not further induced by heregulin. HB-EGF induced tyrosine phosphorylation of only HER4 in SUM44 cells.

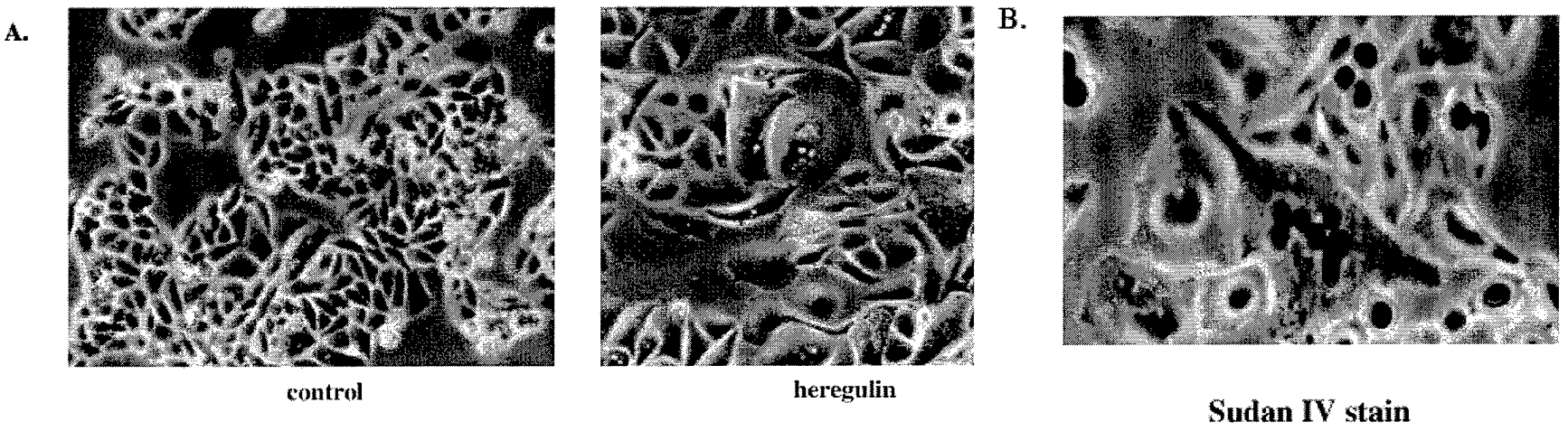



**Figure 11. Differentiation changes in SUM44 cells in response to heregulin.** SUM44 cells were grown in the presence or absence 10ng/ml of heregulin B1 for 1 week and photographed live (A) or after staining with Sudan IV, a neutral lipid stain (B). In the presence of heregulin, cells become larger and flattened, with prominent vacuolization. Sudan IV staining demonstrates lipid droplet formation in heregulin-treated cells.

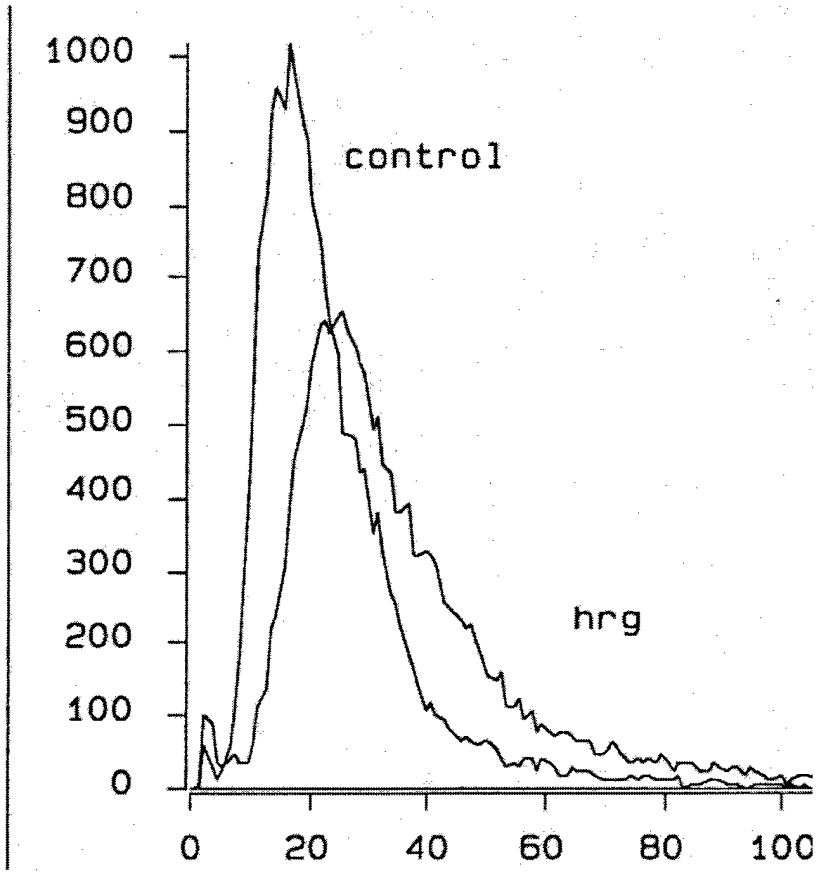



**Figure 12. To quantify the extent of neutral lipid production, cells were stained with a fluorescent neutral lipid stain, Nile Red, and analyzed by FACS.** Treatment with heregulin induces accumulation of neutral lipids, as evidenced by a shift of the curve toward higher intensity staining in the heregulin-treated cells.

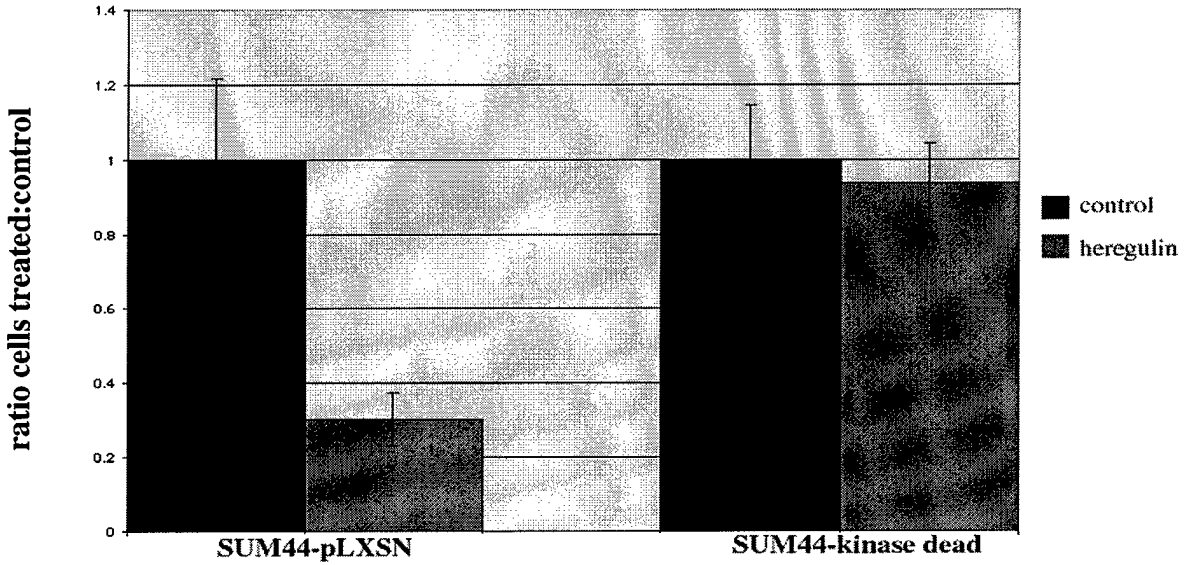



**Figure 13. Manipulation of HER4 in SUM44 cells.** Full-length HER4 containing a mutation in the ATP binding domain which renders it kinase dead (kdHER4) was expressed in SUM44 cells by retroviral infection. After selection in G418, expression was confirmed by RT-PCR. SUM44-kdHER4 cells or vector control cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. Control cells demonstrated growth inhibition in response to heregulin, but this response was abrogated in cells expressing kinase dead HER4.

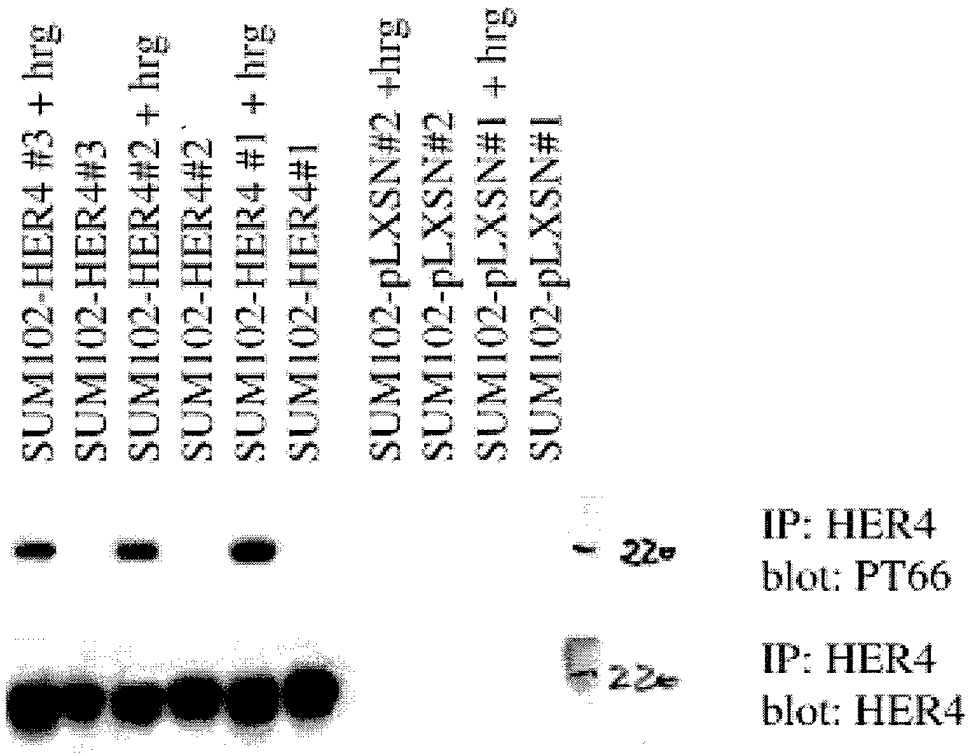



**Figure 14. Stably infected SUM102 cells express HER4 that is activated by heregulin.** Fu ll-length HER4 was stably expressed in SUM102 cells, a human breast cancer cell line that is HER4 negative, by retroviral infection and selection for G418 resistance. HER4 expression was confirmed by western blot using HER4 anti-serum. Vector expression was confirmed in control cells by RT-PCR of neomycin-resistant cells (data not shown). Tyrosine phosphorylation of HER4 in response to heregulin stimulation was measured by immunoprecipitation with anti-HER4 and western blot with anti-phosphotyrosine. In SUM102-HER4 lines, HER4 is not constitutively activated, but activated in response to ligand stimulation.

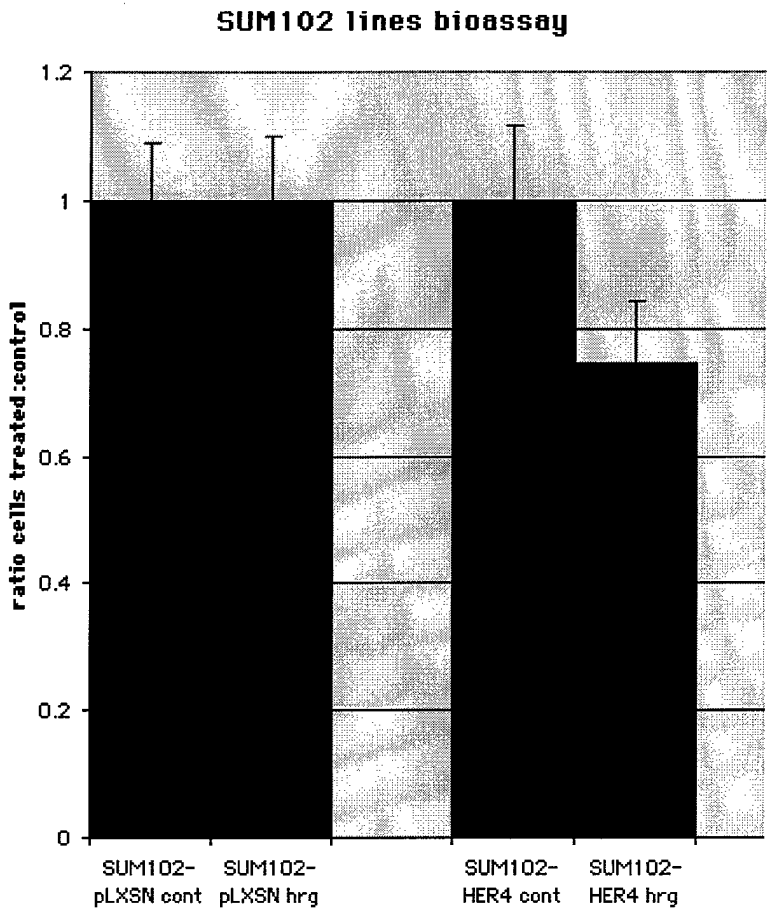



**Figure 15. SUM102 anti-proliferative and differentiative response to heregulin with and without HER4.** SUM102-HER4 or vector control cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. SUM102-HER4 cells are growth inhibited with heregulin, comparable to SUM44 cells, while wild-type and vector control cells do not have an antiproliferative response to HER4.

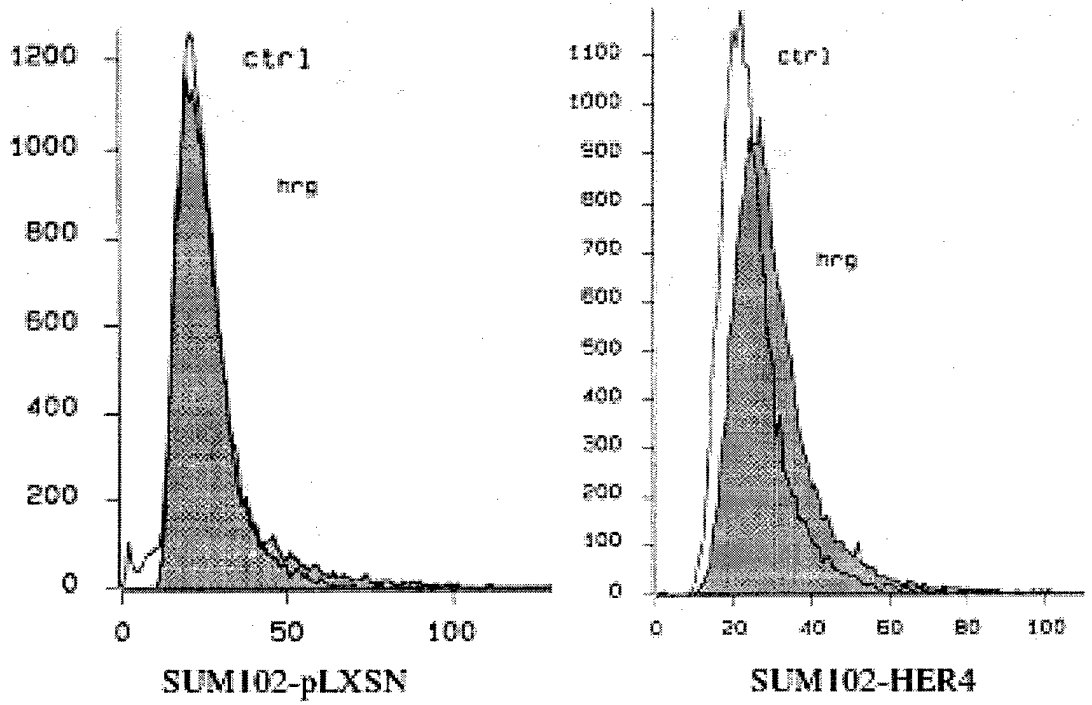



**Figure 16. SUM102 cells expressing vector or HER4 were treated with 10 ng/ml heregulin for 4-6 days, and stained with Nile Red to detect neutral lipids.** Instensity of staining was measured by flow cytometry, and histograms of control and heregulin-treated cells overlaid. SUM102-HER4 cells have increased neutral lipid staining when treated with heregulin, comparable to SUM44 cells, while HER4 negative control cells do not.

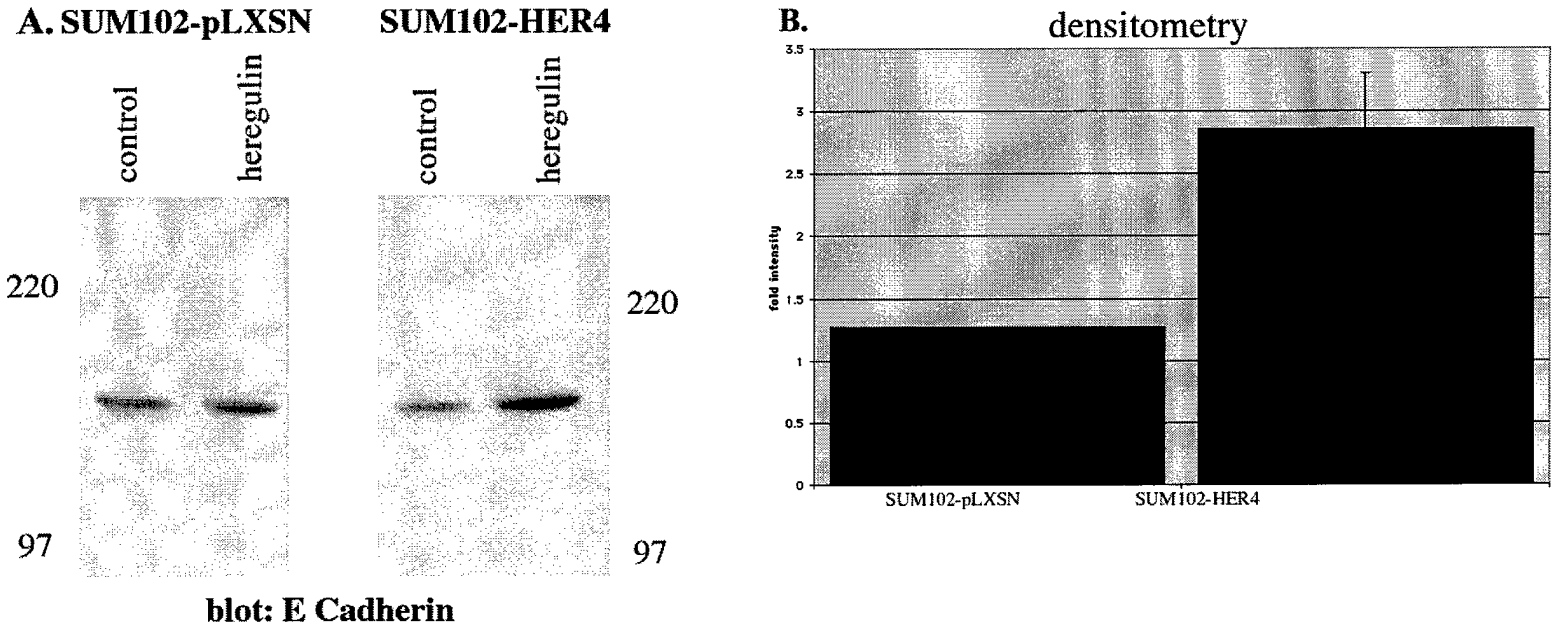



**Figure 17 E-cadherin expression in SUM102-pLXSN vector control cells or SUM102-HER4 cells.** (A) SUM102-pLXSN vector control cells or SUM102-HER4 cells were treated with 10 ng/ml heregulin for 4-6 days, lysed, and western blotting performed with anti-E Cadherin antibody. (B) Densitometry was performed, and standard deviation of at least 3 experiments is shown by the error bars. SUM102-HER4 but not SUM102-pLXSN demonstrated increased expression of E Cadherin in response to heregulin.

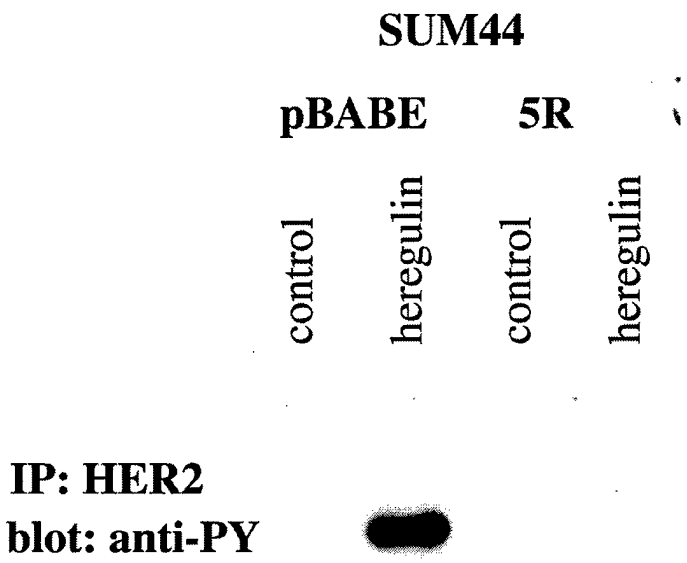



**Figure 18. Effect of intracellular single chain anti-HER2 antibody expression: Abolition of HER2 signaling.** SUM44 cells and SUM102-pLXSN or SUM102-HER4 cells were infected with retrovirus containing vector alone or containing the anti-HER2 endoplasmic reticulum-tagged single chain antibody 5R. After selection in puromycin, removal of HER2 from the membrane by 5R was confirmed by immunohistochemistry, demonstrating loss of HER2 membrane immunoreactivity in the 5R-containing lines (data not shown). Tyrosine phosphorylation of HER2 in response to heregulin stimulation was measured by immunoprecipitation with anti-HER2 and western blot with anti-phosphotyrosine. Cells containing the 5R construct did not have surface HER2 and did not demonstrate HER2 tyrosine phosphorylation, as opposed to cells containing vector control (shown for SUM44 cells). This demonstrates that 5R effectively abrogates HER2 signaling in these cells.

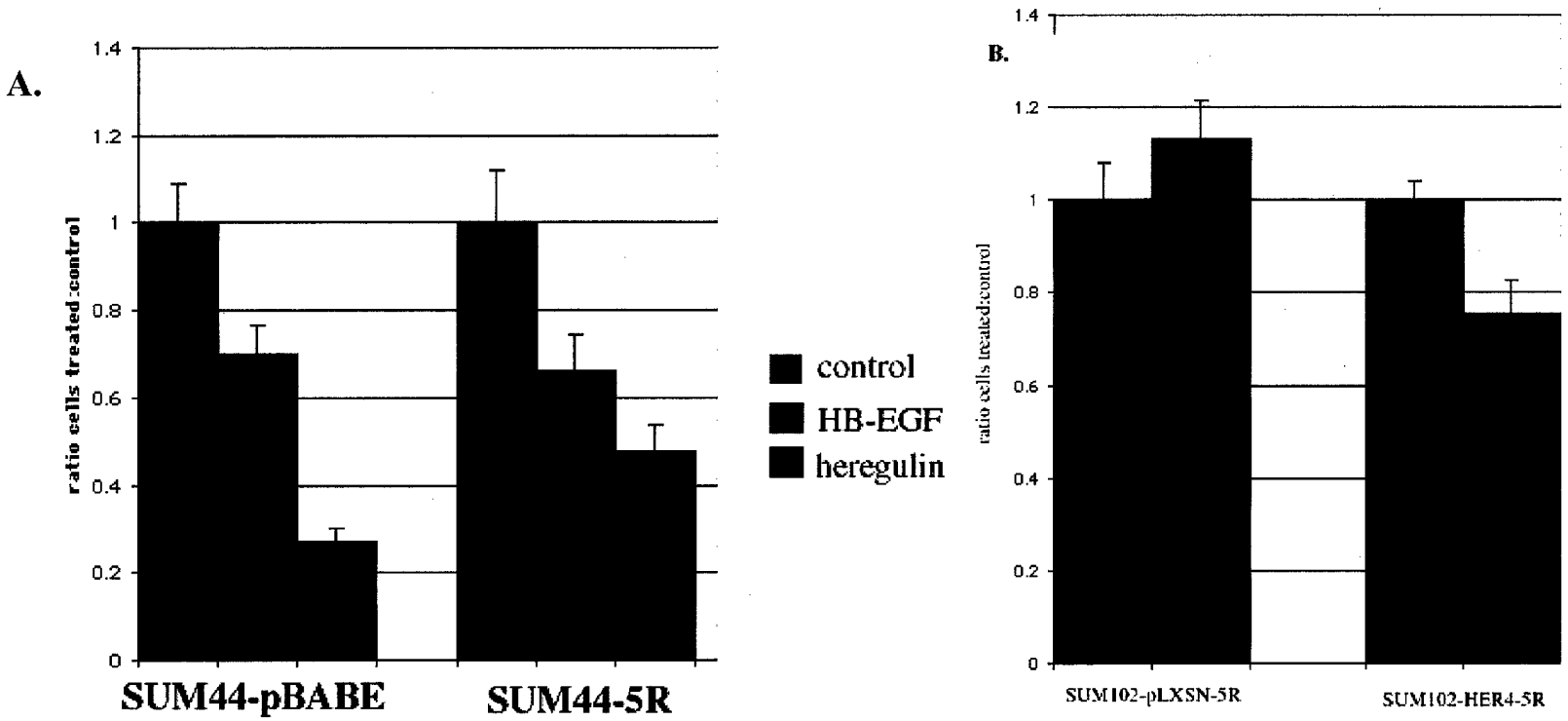
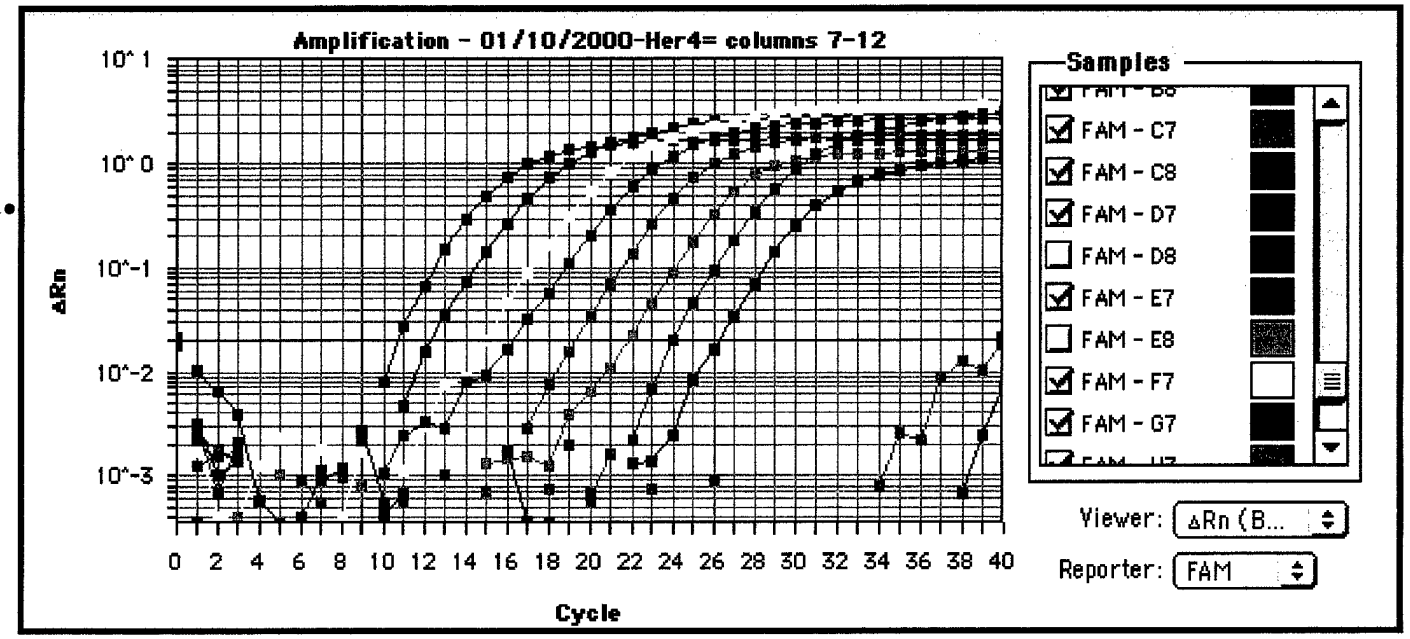



**Figure 19 Anti-proliferative effect of heregulin persists in cells in which HER2 signaling is removed.** (A) SUM44 cells containing vector or 5R (B) SUM102-Her4 cells or vector control cells containing 5R were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 or 100 ng/ml HB-EGF for 3 media changes (7 days), and the number of cells counted. Error bars represent standard deviation of at least 3 experiments. Sequestration of HER2 and removal of HER2 tyrosine phosphorylation did not abrogate the antiproliferative effect of either SUM44 cells or SUM102-HER4 cells. Control SUM102-5R cells (which do not express HER4) did not demonstrate a ligand dependent anti-proliferative effect.

A.

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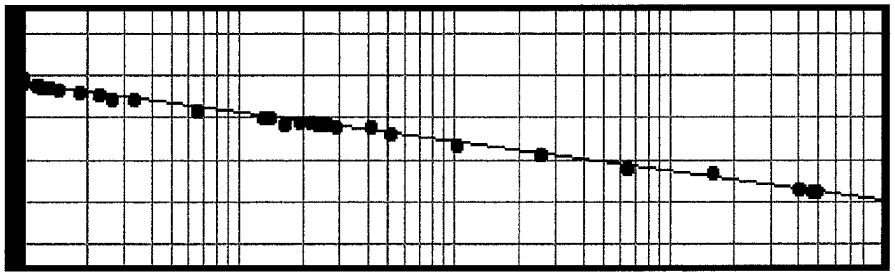

**Figure 20. Amplification plot of HER4 sRNA and standard curve showing HER4 values in various breast cancer cell lines.** (A) ABI 7700 analysis of in vitro transcribed HER4 mRNA. (B) Standard curve and unknowns of HER4 mRNA expression from cell lines. Black dots are standards and red dots are values from various breast cancer cell lines.

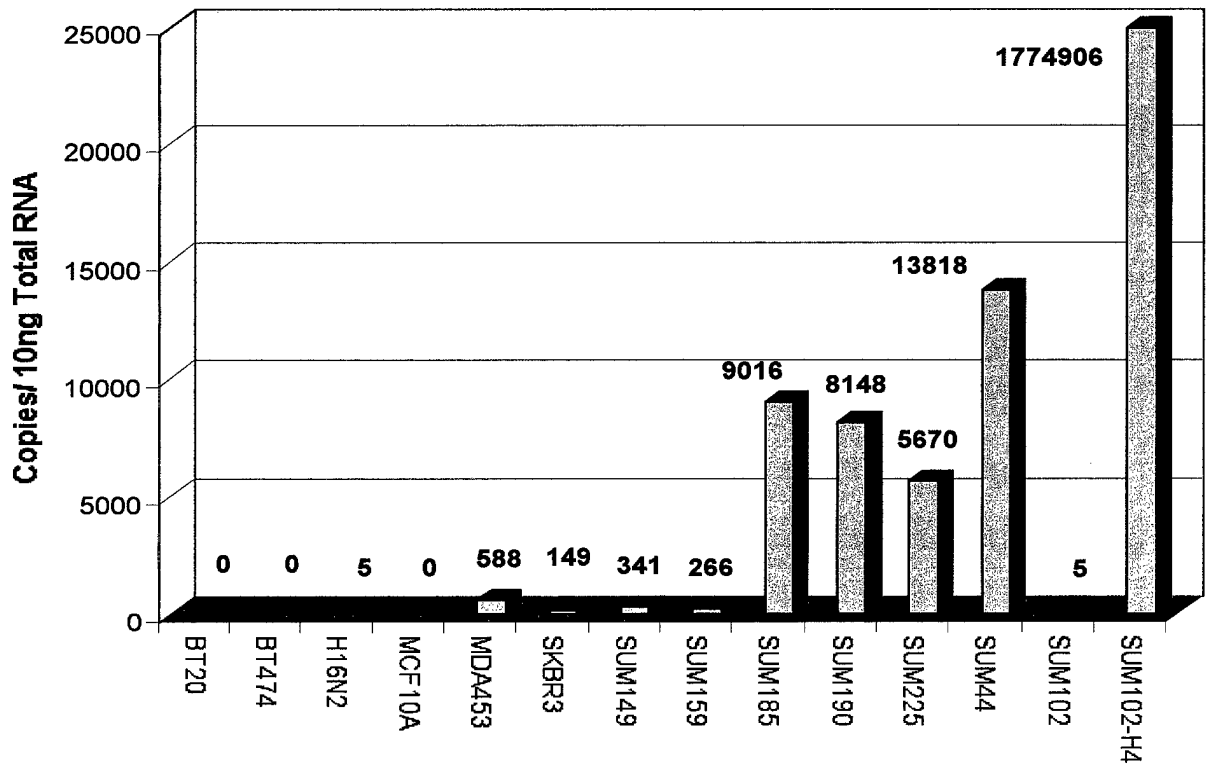


**Figure 21. HER4 copies in breast cancer cell lines.** Total RNA was extracted from cell lines using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of HER4 was determined by real time fluorescence quantitative PCR using HER4 synthetic RNA as a positive control and absolute standard. Values indicate copies of HER4 per 10 ng of total RNA as determined by the standard curve.

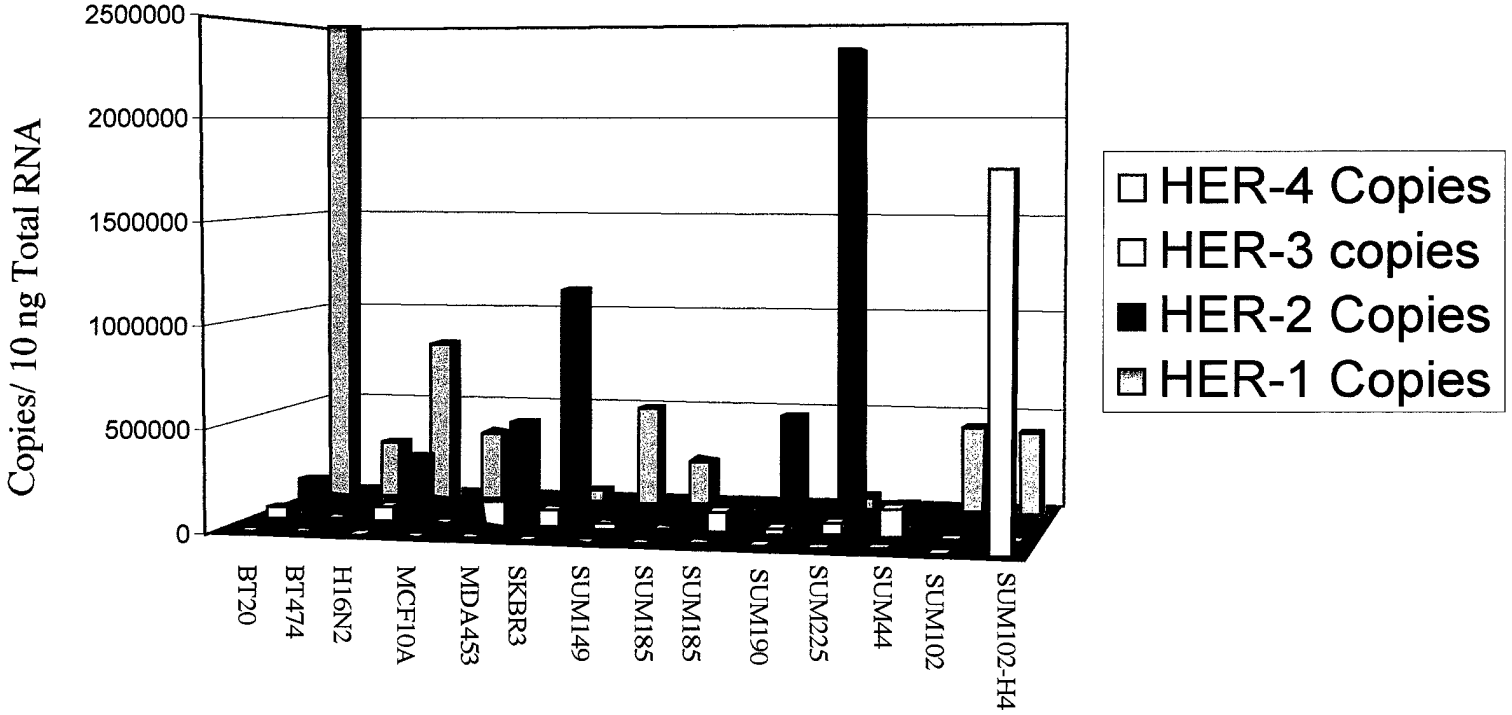


**Figure 22. HER1-4 copies in breast cancer cell lines.** Total RNA was extracted from cell lines using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of HER1-4 was determined by real time fluorescence quantitative PCR using HER1-4 synthetic RNA as positive controls and absolute standards. Copies of HER1-4 per 10 ng of total RNA as determined by standard curves are shown.

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